Accepted Manuscript

Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*

Danival José de Souza, Alain Lenoir, Maria Catarina Megumi Kasuya, Myriam Marques Ramos Ribeiro, Séverine Devers, Joel da Cruz Couceiro, Terezinha Maria Castro Della Lucia

PII: S0889-1591(12)00500-4
DOI: http://dx.doi.org/10.1016/j.bbi.2012.11.014
Reference: YBRBI 2059

To appear in: *Brain, Behavior, and Immunity*

Received Date: 24 July 2012
Revised Date: 21 November 2012
Accepted Date: 22 November 2012

Please cite this article as: de Souza, D.J., Lenoir, A., Kasuya, M.C.M., Ribeiro, M.M.R., Devers, S., Couceiro, J.d.C., Lucia, T.M.C., Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*, *Brain, Behavior, and Immunity* (2012), doi: http://dx.doi.org/10.1016/j.bbi.2012.11.014

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*

Danival José de Souza[1*], Alain Lenoir[3], Maria Catarina Megumi Kasuya[4], Myriam Marques Ramos Ribeiro[2], Séverine Devers[3], Joel da Cruz Couceiro[2], Terezinha Maria Castro Della Lucia[2]

[1*] Corresponding author: Universidade Federal do Tocantins – Campus de Gurupi – Gurupi, 77402-970, TO, Brazil. E-mail: *danivalbr@yahoo.com.br*

[2] Universidade Federal de Viçosa, Departamento de Biologia Animal, Viçosa, 36570-000, MG, Brazil

[3] Université François Rabelais, UMR-CNRS 7261, Faculté des Sciences et Techniques, Tours, 37200, France.

[4] Universidade Federal de Viçosa, Departamento de Microbiologia, Viçosa, 36570-000, MG, Brazil

Running title: de Souza et al.: Ectosymbionts and immunity in *Acromyrmex*

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract

Associations with symbiotic organisms can serve as a strategy for social insects to resist pathogens. Antibiotics produced by attine ectosymbionts (*Actinobacteria*) suppress the growth of *Escovopsis* spp., the specialized parasite of attine fungus gardens. Our objective was to evaluate whether the presence or absence of symbiotic actinobacteria covering the whole ant cuticle is related to differential immunocompetence, respiratory rate and cuticular hydrocarbons (CH). We evaluated these parameters in three worker groups of *Acromyrmex subterraneus subterraneus*: External workers (EXT), internal workers with actinobacteria covering the whole body (INB) and internal workers without actinobacteria covering the whole body (INØ). We also eliminated the actinobacteria by antibiotic treatment and examined worker encapsulation response. INB ants showed lower rates of encapsulation and respiration than did the EXT and INØ ants. The lower encapsulation rate did not seem to be a cost imposed by actinomycetes because the elimination of the actinomycetes did not change the encapsulation rate. Instead, we propose that actinobacteria confer protection to young workers until the maturation of their immune system. Actinobacteria do not seem to change nestmate recognition in these colonies. Although it is known that actinobacteria have a specific action against *Escovopsis* spp., our studies, along with other independent studies, indicate that actinomycetes may also be important for the individual health of the workers.

Key words: Social Immunity; Symbiosis; *Actinobacteria*; Cuticular Hydrocarbons; Energetic Cost

ACCEPTED MANUSCRIPT

1. Introduction

Symbioses play a central role in the evolution of biological complexity and leaf-cutting ants are a prodigious example of this (Ness et al., 2010). More than a century after Belt's suggestion that leaf-cutting ants use leaves to cultivate a fungus (Belt, 1874), other microorganisms were subsequently added to this complex association, including actinomycete bacteria that confer protection against a specialized parasitic fungi of the fungus garden, *Escovopsis* spp. (Currie et al., 1999; Muchovej and Della Lucia, 1990), and black yeasts that compromise the efficiency of bacteria-derived antibiotic defense in fungus-growing ants (Little and Currie, 2008). Additionally, a very large variety of bacteria with an undefined role is found in the nest and in the dump chambers (Scott et al., 2010).

The first studies dealing with *Actinobacteria*-Attini-*Escovopsis* symbiosis revealed a long history of specific coevolution between actinomycetes and *Escovopsis*. However, recent studies have indicated that actinomycete benefits cannot be restricted to protection against *Escovopsis* because antibiotics derived from actinomycetes have a broad spectrum action (Haeder et al., 2009; Sen et al., 2009; Schoenian et al., 2011; Mueller, 2012). Furthermore, considering the myriad of non-specific parasites in the fungus garden, the specificity of antibiotics produced by actinomycetes is improbable.

Actinobacteria are easily detected on the cuticle of the workers because they give a whitish appearance; this led Gonçalves (1961) to suggest that this "strange coating", which is easily removed with needles, was most likely a fungus. Later, Currie et al. (1999) isolated and identified these microorganisms as *Actinobacteria*. They are abundant on workers inside the fungus garden where pathogen control is required to prevent symbiotic fungus collapse. Newly emerged major workers do not seem to carry actinomycetes on the cuticle, but actinomycetes appear on callow workers and progressively increase over time, most likely

ACCEPTED MANUSCRIPT

after transmission by old workers or direct contact with the fungus garden (Poulsen et al., 2003a). In this study, there was an observed growth pattern where major workers were progressively covered by the bacterium a few days after emergence and bacterial cover reached a maximum after 10–15 days.

Actinomycetes are an interesting group of microorganisms because they are responsible for a considerable portion of commercially important bioactive microbial products. Nevertheless, it is not known how actinomycetes influence the ant immune system, although symbiotic microorganisms influence health and disease in animals, and studies have shown that bacteria contribute to their immune defenses. This symbiosis has been observed in various animal taxa: on the amphibian's skin (Becker and Harris, 2010; Woodhams et al., 2007), in the mammalian intestine (Cash et al., 2006) and in insects (de Souza et al., 2009; Oliver et al., 2003). Ants, as well as all other invertebrates, lack an adaptive immune system and must rely on innate immunity as their primary mechanism of defense against parasites and pathogens (Gillespie et al., 1997). Their immune system is closely associated with hemolymph, which consists of cellular (hemocytes) and liquid components (plasma), where the humoral factors are dissolved. Among cellular responses, encapsulation followed by melanization is an efficient innate immune response against infection by parasites (Gillespie et al., 1997) and has been frequently used to evaluate ant immunity (Sorvari et al., 2008; de Souza et al., 2008; de Souza et al., 2009), including that of leaf-cutting ants (Baer et al., 2005; Ribeiro et al., 2011).

Recognition of group members is a critical process to ensure social cohesion within the group. Ants use chemical signatures, composed primarily of cuticular long-chain hydrocarbons, in nestmate recognition (d'Ettorre and Lenoir, 2010). To protect the colony against parasites, it is expected that workers can discriminate nestmates based on individual immunological state. Likewise, odor perception can be affected by immune response. For

ACCEPTED MANUSCRIPT

example, when honeybee immune systems are triggered by the non-pathogenic immunogenic elicitor lipopolysaccharide (LPS), they have a reduced ability to associate an odor with a sugar reward (Mallon et al., 2003). Plenty of bacteria have been shown to play an important role in the production of volatile compounds, some of which may act as chemicals messengers within or between species (Leroy et al., 2011). Currently, the role of actinomycetes in chemical communication is unknown and requires more investigation.

One general attribute of immune functions is that their operation requires resources that the host might have used for another function (Sheldon and Verhulst, 1996). Immune stimulation increases energy consumption (Freitak et al., 2003; Tyler et al., 2006) and decreases longevity in insects (Armitage et al., 2003). Thus, considering that the immune system is costly to develop, maintain or activate, ants that invest less in immune defense can direct energy to other activities, such as fungus garden care or brood care. If ectosymbiotic bacteria provide immune protection for the ants, the ants can stay protected even with a less active immune system. Inferences on the energetic cost of physiological processes in insects can be made by the evaluation of the oxygen consumption rate, which has been studied in leaf-cutting ants (Hebling-Beraldo and Mendes, 1981; Hebling et al., 1992; Poulsen et al., 2003a).

Our objectives were to evaluate whether the presence or absence of symbiotic bacteria covering the ant cuticle is related to differences in (1) the encapsulation responses between workers, (2) the level of metabolic activity, which is determined by measuring individual respiratory rates, and (3) the cuticular hydrocarbons pattern. We also eliminated the bacteria using an antibiotic treatment and examined worker encapsulation response after the treatment.

ACCEPTED MANUSCRIPT

2. Material and Methods

2.1. Colony maintenance

In this study, we used adult colonies of *Acromyrmex subterraneus subterraneus* that had been collected three years before in Viçosa, Minas Gerais State, Brazil. The colonies were maintained in the laboratory at the Animal Biology Department Insectary at the Federal University of Viçosa, according to the methodology developed by Della Lucia et al. (1993). They consisted of a vial (3 L) with the fungus garden connected to a foraging arena and were maintained at 25 ± 2 ºC with a relative humidity of 75 ± 5% and a 12:12 light:dark regime. On a daily basis, the ants received fresh leaves of *Ligustrum japonicum* Thumb, *Tecoma stans* L., *Acalypha wilkesiana* Müll Arg and *Rosa* spp., in addition to clean water.

2.2. Workers selection and encapsulation rate assay

The encapsulation response depends on humoral and cellular factors, and the cellular defense system is coupled with humoral defense in the melanization of pathogens. Thus, the encapsulation rate assay provides an accurate measure of immunocompetence, which is defined as the ability to produce an immune response (Ahtiainen et al., 2004; Rantala and Kortet, 2004). We used three three-year-old colonies (A, B, and C) for measuring the encapsulation rate of *A. subterraneus subterraneus* workers. Three groups of workers of similar size (approximately 2.4 mm of head capsule width) were defined based on their nest location (internal/external) and the extent of actinomycetes covering their cuticle (clearly visible/not visible): (1) external workers without visible bacteria covering the body (EXT), (2) internal workers with bacteria covering the whole body (INB) and (3) internal workers without visible bacteria covering the whole body (INØ). Considering the wide variation in bacterial coverage of the ants, we have chosen two distinct worker classes. INB workers

ACCEPTED MANUSCRIPT

referred to those whose head, thorax and gaster were entirely covered with bacteria from a top view. This pattern corresponds to 'score 12' (maximum) established and used by Poulsen et al. (2003a). From a top view, the EXT and INØ workers exhibited no coverage of bacteria on the head, thorax and abdomen. Insertion of an artificial antigen in the hemocoel provokes its encapsulation, and this method has been frequently used to evaluate insect immunity (de Souza et al., 2009; de Souza et al., 2008; Fytrou et al., 2006; Lu et al., 2006; Sorvari et al., 2008; Vainio et al., 2004). We measured the encapsulation response by inserting an inert antigen, a 1.5 mm-long piece of a sterile nylon monofilament (0.12 mm diameter), into each ant's thorax between the second and third leg pairs. After introduction of the antigen, the workers were individually placed in glass test tubes. The tubes were maintained in an incubator at 25 °C, 75% R. H., in the dark. This procedure was carried out on 10 workers from each colony, with a total of 30 workers for each group. Twenty-four hours later, the implants were removed from the hemocoel and placed on a glass slide to be mounted in Entellan© medium. Nylon monofilament was examined under a light microscope and photographed using a digital camera (Axioskop 40 Zeiss microscope). The mean gray value of the whole implant was measured using the ImageJ 1.37v software. It was assumed that the darkest gray received the highest encapsulation rate (total black). The background gray value was subtracted to correct the gray values of the implants. The colony was included as a random factor and treatments were analyzed by an ANOVA followed by an Unequal N HSD test at 5% probability.

2.3. Antibacterial treatment and encapsulation response

In this experiment, we used a fourth colony (colony D) to test the effects of removing bacteria on worker immunity. To kill the bacteria, we followed the methodology described by Poulsen et al. (2003a). We established six experimental treatments using workers with bacteria covering the whole body: (1) 22 without treatment, (2) 20 treated with a dry brush to

ACCEPTED MANUSCRIPT

remove their bacterial cover, (3) 20 treated with a wet (water only) brush, (4) 20 treated with a brush containing a solution of penicillin G (622 mg/L), (5) 20 with a brush containing a solution of streptomycin sulfate (1230 mg/L) and (6) 20 treated with a brush containing a mixture of the two antibiotics. Ant workers were all about the same size (~ 2.4 mm HW) and the brushing operation lasted approximately 10 s. Afterwards, all ants were marked with a dot of paint and placed in mini-colonies established in plastic pots containing 100 mL of fungus garden and approximately 100 nestmate workers without visible bacteria coating. Ten days later, the marked workers were removed for an encapsulation assay, as described in section 2.2. We verified that these marked workers did not show a visible white coating of bacteria in the integument, confirming that the treatments were effective. The groups were compared by an ANOVA followed by an Unequal N HSD test at 5% probability.

2.4. Respirometry assay

The aim of this study was to assess the metabolic rate and to infer a possible energetic cost of maintaining ectosymbiotic bacteria. The production of carbon dioxide was measured in a carbon dioxide analyzer (TR 2; Sable System International, Las Vegas, Nevada, USA) using methods adapted from Hebling et al. (2000) and Guedes et al. (2006). A series of 25 mL flasks was used, each flask containing three workers (2.4 mm head capsule width) from each group (EXT, INB, and INØ) in a completely closed system. Carbon dioxide-free air was injected into the flasks for 2 min at 600 mL/min. An infrared reader was connected to the outlet of the system to quantify carbon dioxide (µmol). The test tubes were connected to the system for three hours before measurement of CO_2 production from the workers, which was achieved by injection of CO_2-free air into the vials for two minutes at a flow rate of 600 mL/min. This air flow directs CO_2 to an infrared reader connected to the system and allows rapid quantification of the amount of CO_2 produced on an hourly basis (in µmol). There were 14 replicates for each group, which were taken at the same proportion from three colonies (A,

ACCEPTED MANUSCRIPT

B, and C). In total, we took 42 workers from each colony. The value of CO_2 production for each vial was divided by three to calculate the mean respiratory rates, which were analyzed by an ANOVA followed by a Tukey test at 5% significance and using Statistica 7.0. The colony was included as a random factor.

2.5. Chemical analyses

In this experiment, we used the same three colonies (A, B and C). The head-thorax with the legs taken from the three groups of media workers (EXT, INB and INØ); 6 workers per group per colony were immersed in 1 mL of pentane and removed after 30 min. Before analysis, the solvent was evaporated and redissolved with 5 µL of pentane; we then added 2 µL of pentane containing 200 ng of eicosane (C20) as an internal standard. Two microliters were injected into a FID gas chromatograph (VGM250Q system, Perkin-Elmer) using a DB-5 fused silica capillary column. The temperature was maintained at 150 °C during the splitless initial two minutes, raised from 150 °C to 310 °C at 5 °C/min and held at 310 °C for the last 10 min. The cuticular hydrocarbons were previously identified (Viana, 1996; Viana et al., 2001), and to verify the names of the peaks, including the smaller peaks, we analyzed in more detail the cuticular profile with the same GC coupled to a Perkin-Elmer MS operating 70 EV. We used a high-temperature column (DB-5HT, 30 m, 0.251 mm x 0.10 µm) with the same temperature program. The areas of the peaks were estimated by peak integration using a TurboChrome Workstation. From the area, we calculated the quantities and relative proportions of substances using the internal standard area (ng per sample). The relative proportions of CHs were used to construct a dendrogram. The total quantities of hydrocarbons were compared with a Kruskal-Wallis test. The profiles between the three groups were compared with a dendrogram using the single-link Ward method and Euclidian distance. We also verified that there were no differences between the colonies.

ACCEPTED MANUSCRIPT

Because products of bacterial metabolism may contribute to the colony odor and play an important role in nestmate recognition (see for termites (Matsuura, 2001) and Minkley et al. (2006)), we analyzed whether the hydrocarbons could have originated from actinobacteria. A *Pseudonocardia* strain (GenBank accession code JF514546; the other two isolates were JX543365 and JX543366) was isolated from *A. subterraneus subterraneus* workers (see Appendix A for the isolation and identification of the bacterium), and we performed a pentane extraction from a small piece of a 1 cm diameter of an agar pure culture that was analyzed as previously described. We also analyzed the hydrocarbons on the gelose used for bacteria culture in the same chromatographic conditions.

3. Results

3.1. Bacteria and encapsulation rate

Variation was observed in the encapsulation rate among the three groups of workers ($F_{2, 81} = 35.66$, $P < 0.001$), i.e., there was a significant effect of treatment on the encapsulation response. Internal workers with bacteria (INB) had the lowest encapsulation rate compared with internal workers without bacteria (INØ) and external workers (EXT) (Unequal N HSD, $P < 0.05$). The colonies showed a variation in the degree of encapsulation ($F_{2, 81} = 16.62$, $P < 0.001$), but no interaction between treatments and colonies was verified ($F_{4, 81} = 0.82$, $P = 0.52$); the three colonies exhibited the same pattern of encapsulation rate variation (Fig. 1).

3.2. Bacteria removing and encapsulation rate

The encapsulation rates of workers whose actinobacteria were removed by streptomycin or a combination of streptomycin + penicillin were reduced in comparison with control workers, brush-treated or penicillin-treated workers (Fig. 2). Ten days after treatment, we could verify that the treatment had a highly significant effect ($F_{5, 72} = 8.92$, $P < 0.001$). We

ACCEPTED MANUSCRIPT

compared the survival proportion of the ants undergoing the bacteria removal treatments against that observed in the control groups. The hypothesis tested was H0: *P* control = *P* treatment vs. H1: *P* control > *P* treatment (one-sided test). The p-value is computed based on the t-value for the following comparisons: Control vs. Dry brush, *P* = 0.0042; Control vs. Wet brush, *P* = 0.0001; Control vs. Pen. G, *P* = 0.0021; Control vs. Strep., *P* = 0.0021; Control vs. Pen. G + Strep., *P* = 0.0002. As all treatments provoked mortality in treated ants, including the Dry brush, it appears that ant mortality is due to the stress of the ant removal from the nest and its manipulation. It is possible that the treatments to eliminate actinobacteria cause selective survival; therefore, we would be sampling the encapsulation response of a subset of the ants. However, we have no evidence of differential mortality associated with the level of encapsulation response because similar mortality occurred in groups with higher encapsulation response (Wet brush) and in groups with lower encapsulation response (Pen. G + Strep.), as verified in Fig. 2.

3.3. Respiratory rate

The individual metabolic rate of the workers, measured in terms of CO_2 production, showed a pattern of increase as workers lost their bacterial coating and switched to external activities (Fig. 3; Kruskal-Wallis, H (2 n = 42) = 6.94, *P* = 0.03). Individuals living inside the nest, with or without a whitish coat of bacteria, had significantly lower respiration rates compared with individuals performing external activities.

3.4. Hydrocarbons

Hydrocarbon quantities on the thorax did not vary among the three groups: 119.8 ± 27.7 ng per ant (mean ± SE) for EXT, 81.1 ± 11.0 for INØ and 132.3 ± 32.8 for INB (Kruskal-Wallis H (2, n = 53) = 1.67, *P* = 0.43) (See Fig. S1).

ACCEPTED MANUSCRIPT

The hydrocarbon profile was simple (24 peaks, see Fig. S2). The hydrocarbons observed were mainly methyls (11-+13-+15-MeC29, more than 30%, see table S1; 11-+13-MeC31 -10%) and the corresponding dimethyls (respectively 11,15-+13,17-DiMeC29, 5% and 11,15-+13,17-DiMeC31, 6%), and the hydrocarbon profile was not changed according to the ant group. In the dendrogram, the samples were mixed in arbitrary groups (see Fig. S3).

We found some of the ant hydrocarbons in the bacteria and also in the gelose (see Table S1), but in very small quantities (4.5 and 9.7 ng, respectively). These hydrocarbons were all present on the ant's cuticle.

4. Discussion

The encapsulation rate of *Acromyrmex subterraneus subterraneus* workers with a visible actinobacteria coating was significantly lower than that of workers without bacteria. It seems that ectosymbionts are not responsible for reducing this immune response because their removal did not increase the encapsulation response. Instead, the results suggest that actinobacteria could give protection to young workers until maturation of their immune system. We affirm that internal workers with bacteria are younger and external workers older; this conclusion is based (*i*) on our daily observation of laboratory colonies, which included several *Acromyrmex* species, and (*ii*) on the studies conducted by Poulsen et al. (2003a) in *Acromyrmex octospinosus*. Moreover, temporal polyethism is ubiquitous in social insect colonies. Newly emerged workers perform tasks within the nest, such as brood care and nest maintenance, and progress to tasks outside as they age (Wilson, 1971). Recently, it has been demonstrated that *Actinobacteria* constitute a line of defense against entomopathogenic fungi in Attini ants (Mattoso et al. 2012). These authors verified that experimental removal of the bacterial coating after antibiotic treatment increased the susceptibility of *A. subterraneus subterraneus* workers to infection by the entomopathogenic fungus *Metarhizium anisopliae*. This study offered direct evidence for the benefits of actinobacteria ectosymbionts to the

ACCEPTED MANUSCRIPT

health of the workers. We are also conducting experiments to evaluate the action of an actinomycete isolate from *A. subterraneus subterraneus* against entomopathogenic fungi isolate from the same ant species. Preliminary results have shown inhibitory effects of the actinomycete against the entomopathogenic fungus *Aspergillus ochraceus.*

The variation of encapsulation rate between the groups is not a function of worker location because the encapsulation rate of internal workers without actinobacteria is similar to that of external workers without actinobacteria. Consistent with our studies, Armitage and Boomsma (2010) have found a significant increase in phenoloxydase activity (an enzyme involved in melanization) in older workers of *A. octospinosus*. Our results, coupled with the studies of Armitage and Boomsma (2010), highlight a pattern of increasing immunity as *Acromyrmex* workers age.

Different attine ant species can use different strategies against pathogens. For example, workers of *Atta*, another leaf-cutting ant genus, do not have visible actinobacteria and completely lost the cuticular structures to rear actinomycetes (Mueller *et al*., 2008). In *Atta sexdens rubropilosa*, workers performing internal activities had a higher encapsulation rate than those working outside the colony, which is different from what we observed for *A. subterraneus subterraneus* (Ribeiro et al., 2011). Comparative studies of immune response among species, differing in the presence or absence of bacteria coating, could determine whether ectosymbionts act to modulate innate immune responses in attine ants.

Actinomycetes seem to combat primarily *Escovopsis* spp., but inhibitory effects of lower intensity have been demonstrated against other fungi, including entomopathogenic fungi (Haeder et al., 2009). Under more vulnerable conditions, where the immune system of younger workers is less active, actinobacteria may offer protection against pathogens. It has been demonstrated that other insects can be protected by symbiotic actinobacteria against

ACCEPTED MANUSCRIPT

pathogens, parasitoids or predators. The actinomycetes' ability to produce a wide range of secondary metabolites, including several with antibiotic properties, partially accounts for this trend in insect-actinomycetes symbioses (Kaltenpoth, 2009).

From *Hydra* to humans, we can find examples of epithelia selecting the bacterial community to live on them (Fraune and Bosch, 2010). In Attini ants, actinomycetes live in specialized structures that are elaborate cuticular crypts with associated exocrine glands (Currie et al., 2006). Their abundance is age-dependent, and their dependence on metapleural gland secretion supports the hypothesis of active mechanisms regulating their presence (Poulsen et al., 2003b). Thus, another hypothesis to be tested consists of verifying an increase of ectosymbionts when the workers are immunocompromised.

In our study, external workers exhibited a more elevated respiratory rate than did workers with actinobacteria. Although it is not possible to separate the fraction of energy due to the presence actinomycetes, it is at least evident that actinomycetes do not pose a high energy cost to workers. Our data support a pattern of increase of metabolic rate as *Acromyrmex* workers age and their immune system achieves maturation, and at this point, they are able to perform external activities.

Actinobacteria do not change the cuticular profile or the hydrocarbon quantities of the host ant; this is in contrast to the fungus symbiont, which is important in colonial recognition (Viana and Lenoir, 1996). This indicates that nestmate recognition is not modified, which was expected because foragers and some internal ants do not have the actinobacteria. Workers with and without ectosymbionts cannot be discriminated based on cuticular odors. Some hydrocarbons found on the actinobacteria culture may be general for all bacteria membranes and may have contaminated the gelose. Hydrocarbon production is very low and most likely is not important compared to ant cuticle production, indicating that the ant cuticular

ACCEPTED MANUSCRIPT

hydrocarbons do not originate from the actinobacteria. Nevertheless, actinobacteria also produce some hydrocarbons that may be a signal for recognition by ants, as Zhang et al. (2007) have recently shown that workers are able to recognize their own bacterial strain.

Our studies, along with other independent studies, suggest a possible new role of actinomycetes for the leaf-cutting ants, thus reinforcing emerging views that integumental biofilms protect ants primarily against ant diseases (Mueller, 2012). Considering that the combat of infectious diseases is a major challenge for large insect societies, actinomycetes may ensure protection to younger attine ants until the maturation of their immune system, and this protection is achieved with low energetic cost.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Aline Mello and Mr. Alberto Soares Corrêa for technical assistance. This work was financially supported by CNPq-Brazil, French CNRS and François Rabelais University of Tours.

ACCEPTED MANUSCRIPT

5. References

Ahtiainen, J. J., Alatalo, R. V., Kortet, R., Rantala, M., 2004. Sexual advertisement and immune function in an arachnid species (Lycosidae). Behavioral Ecology, 15, 602-606.

Armitage, S.A.O., Thompson, J.J.W., Rolff, J., Siva-Jothy, M.T., 2003. Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. Journal of Evolutionary Biology, 16, 1038-1044.

Armitage, S. A. O., Boomsma, J. J., 2010. The effects of age and social interactions on innate immunity in a leaf-cutting ant. Journal of Insect Physiology, 56, 780-787.

Baer, B., Krug, A., Boomsma, J. J., Hughes, W. O. H., 2005. Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection. Insect Sociaux, 52, 298-303.

Becker, M.H., Harris, R.N., 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS ONE, 5, e10957.

Belt, T., 1874. The naturalist in Nicaragua. E. Bumpus, London.

Cash, H.L., Whitham, C.V., Behrendt, C.L., Hooper, L.V., 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science, 313, 1126-1130.

Currie, C.R., Mueller, U.G., Malloch, D., 1999. The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Sciences of the United States of America, 96, 7998-8002.

Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J., 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science, 311, 81-83.

Della Lucia, T.M.C., Vilela, E.F., Anjos, N. & Moreira, D.D.O. 1993. Criação de formigas cortadeiras em laboratório. In: As Formigas Cortadeiras (Ed. por T.M.C. Della Lucia), pp. 151-162. Viçosa: Folha de Viçosa.

ACCEPTED MANUSCRIPT

de Souza, D., Bezier, A., Depoix, D., Drezen, J.-M., Lenoir, A., 2009. *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. BMC Microbiology, 9, 29.

de Souza, D.J., Van Vlaenderen, J., Moret, Y., Lenoir, A., 2008. Immune response affects ant trophallactic behaviour. Journal of Insect Physiology, 54, 828-832.

d'Ettorre, P., Lenoir, A., 2010. Nestmate recognition, in: Lach, L., Parr, C., Abbott, K. (Eds.), Ant ecology. Oxford University Press, Oxford, pp. 194-208.

Fraune, S., Bosch, T.C.G., 2010. Why bacteria matter in animal development and evolution. Bioessays, 32, 571-580.

Freitak, D., Ots, I., Vanatoa, A., Hõrak, P., 2003. Immune response is energetically costly in white cabbage butterfly pupae. Proceedings of the Royal Society of London, Series B: Biological Sciences, 270, S220-S222.

Fytrou, A., Schofield, P.G., Kraaijeveld, A.R., Hubbard, S.F., 2006. *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. Proceedings of the Royal Society of London, Series B: Biological Sciences, 273, 791-796.

Gillespie, J.P., Kanost, M.R., Trenczek, T., 1997. Biological mediators of insect immunity. Annual Review of Entomology, 42, 611-643.

Gonçalves, C.R., 1961. O gênero *Acromyrmex* no Brasil (Hym. Formicidae). Studia Entomologica, 4, 113-180.

Guedes, R.N.C., Oliveira, E.E., Guedes, N.M.P., Ribeiro, B., Serrão, J.E., 2006. Cost and mitigation of insecticide resistance in the maize weevil, *Sitophilus zeamais*. Physiological Entomology, 31, 30-38.

Haeder, S., Wirth, R., Herz, H., Spiteller, D., 2009. Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. Proceedings of the National Academy of Sciences, 106, 4742-4746.

ACCEPTED MANUSCRIPT

Hebling-Beraldo, M. J. A., Mendes, E. G., 1981. The respiratory metabolism of the castes of two leaf-cutting ants, *Atta laevigata* (F. Smith 1858) and *Atta sexdens rubropilosa* Forel 1908. Comparative Biochemistry and Physiology A – Comparative Physiology, 68, 241-247.

Hebling, M.J.A., C.H.S. Penteado & E.G. Mendes. 1992. Respiratory regulation in workers of the leaf cutting ant *Atta sexdens rubropilosa* Forel 1908. Comparative Biochemistry and Physiology A - Comparative Physiology 101: 319-322.

Hebling, M.J.A., Bueno, O.C., Pagnocca, F.C., Silva, O.A.d., Maroti, P.S., 2000. Toxic effects of *Canavalia ensiformis* L. (Leguminosae) on laboratory colonies of *Atta sexdens* L. (Hym., Formicidae). Journal of Applied Entomology, 124, 33-35.

Kaltenpoth, M., 2009. *Actinobacteria* as mutualists: general healthcare for insects? Trends in Microbiology, 17, 529-535.

Leroy, P. D., Sabri, A., Verheggen, F. J., Francis, F., Thonart, P., Haubruge, E., 2011. The semiochemically mediated interactions between bacteria and insects. Chemoecology, 21, 113-122.

Little, A.E.F., Currie, C.R., 2008. Black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. Ecology, 89, 1216-1222.

Lu, J.F., Hu, J., Fu, W.J., 2006. Levels of encapsulation and melanization in two larval instars of *Ostrinia furnacalis* Guenee (Lep., Pyralidae) during simulation of parasitization by *Macrocentrus cingulum* Brischke (Hym., Braconidae). Journal of Applied Entomology, 130, 290-296.

Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune response inhibits associative learning in insects. Proceedings of the Royal Society of London, Series B: Biological Sciences, 270, 2471-2473.

ACCEPTED MANUSCRIPT

Mattoso, T.C., Moreira, D. D. O., Samuels, R. I., 2012. Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. Biology Letters, 8, 461-464.

Matsuura, K., 2001. Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos, 92, 20-26.

Minkley, N., Fujita, A., Brune, A., Kirchner, W., 2006. Nest specificity of the bacterial community in termite guts (*Hodotermes mossambicus*). Insectes Sociaux, 53, 339-344.

Muchovej, J.J., Della Lucia, T.M., 1990. *Escovopsis*, a new genus from leaf cutting ant nests to replace *Phialocladus nomem invalidum*. Mycotaxon, 37, 191-195.

Mueller, U. G., Dash, D., Rabelin, C., Rodrigues, A., 2008. Coevolution between attine ants and actinomycete bacteria: A reevaluation. Evolution, 62, 2894–2912.

Mueller, U. G., 2012. Symbiont recruitment versus ant-symbiont co-evolution in the attine ant–microbe symbiosis. Current Opinion in Microbiology, 15, 269-277.

Ness, J., Mooney, K., Lach, L., 2010. Ant as mutualists, in: Lach, L., C., P., Abbott, K. (Eds.), Ant ecology. Oxford University Press, Oxford, pp. 97-114.

Oliver, K.M., Russell, J.A., Moran, N.A., Hunter, M.S., 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences, 100, 1803-1807.

Poulsen, M., Bot, A.N.M., Currie, C.R., Nielsen, M.G., Boomsma, J.J., 2003a. Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. Functional Ecology, 17, 260-269.

Poulsen, M., Bot, A.N., Boomsma, J.J., 2003b. The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. Naturwissenschaften, 90, 406-409.

ACCEPTED MANUSCRIPT

Rantala, M., Kortet, R., 2004. Male dominance and immunocompetence in a field cricket. Behavioral Ecology, 15, 187-191.

Ribeiro, M. M. R., de Souza, D. J., Gandra, L. C., Della Lucia, T. M. C., 2011. Immunocompetence and energetic metabolism in different groups of workers of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). Sociobiology, 58, 207-216.

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H., Spiteller, D., 2011. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. Proceedings of the National Academy of Sciences, 108, 1955-1960.

Scott, J.J., Budsberg, K.J., Suen, G., Wixon, D.L., Balser, T.C., Currie, C.R., 2010. Microbial community structure of leaf-cutter ant fungus gardens and refuse dumps. PLoS ONE, 5, e9922.

Sen, R., Heather, D. I., Estrada, D., Dowd, S. E., Hong, E. Mueller, U. G., 2009. Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. Proceedings of National Academy of Science of USA, 106, 17805-17810.

Sheldon, B. C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology and Evolution, 11, 317-321.

Sorvari, J., Hakkarainen, H., Rantala, M.J., 2008. Immune defense of ants is associated with changes in habitat characteristics. Environmental Entomology, 37, 51-56.

Tyler, E., Adams, S., Mallon, E., 2006. An immune response in the bumblebee, *Bombus terrestris*, leads to increased food consumption. BMC Physiology, 6, 6.

Vainio, L., Hakkarainen, H., Rantala, M., Sorvari, J., 2004. Individual variation in immune function in the ant *Formica exsecta*; effects of the nest, body size and sex. Evolutionary Ecology, 18, 75-84.

ACCEPTED MANUSCRIPT

Viana, A.M., 1996. La reconnaissance coloniale du couvain et du champignon chez la fourmi champignonniste *Acromyrmex subterraneus subterraneus*. Université Paris XIII, Villetaneuse, France, 136 p.

Viana, A.M.M., Frezard, A., Malosse, C., Della Lucia, T.M.C., Errard, C., Lenoir, A., 2001. Colonial recognition of fungus in the fungus-growing ant *Acromyrmex subterraneus subterraneus* (Hymenoptera : Formicidae). Chemoecology, 11, 29-36.

Viana, A.M.M., Lenoir, A., 1996. Colonial fungus recognition of the attine ant *Acromyrmex subterraneus* (Hymenoptera Formicidae). Proceedings of the 20th International Congress of Entomology, Firenze, Italy, abstract p. 402.

Wilson, E. O., 1971. The insect societies, Harvard University Press, Cambridge.

Woodhams, D.C., Vredenburg, V.T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C.J., Rollins-Smith, L.A., Harris, R.N., 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biological Conservation, 138, 390-398.

Zhang, M.M., Poulsen, M., Currie, C.R., 2007. Symbiont recognition of mutualistic bacteria by *Acromyrmex* leaf-cutting ants. ISME Journal, 1, 313-320.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure Captions

Fig. 1 – Encapsulation rate (darkness value of implant) in three groups of workers of *Acromyrmex subterraneus subterraneus*. Superscript letters indicate significant differences among the groups, not among the colonies (Unequal N HSD, $P < 0.05$). The numbers inside each column indicate the n value. (Mean ± SE)

Fig. 2 - Encapsulaton rate (darkness value of implant) of workers with bacterial coating after five treatments to remove actinomycetes and control. Superscript letters indicate significant differences among the groups (Unequal N HSD, $P < 0.05$). The numbers inside each column indicate the n value. (Mean ± SE)

Fig. 3 – Respiration rate (µL CO_2/h) per ant; (median ± semi-interquartile range; n = 14) of workers from three colonies (A, B and C). Superscript letters indicate significant differences among the groups. KKW ANOVA (H (2, N= 42) =6.94, P =.031) followed by multiple comparisons test.

Appendix

Table S1 – Relative hydrocarbon quantities (mean % ± SE) of the three worker categories (INB, INØ and EXT), all workers, actinobacteria and gelose used for actinobacteria culture.

Fig. S1. Cuticular hydrocarbon quantities (head + thorax + legs) mean per worker ± SE.

Fig. S2. – Chromatogram of *Acromyrmex subterraneus subterraneus* workers. Numbers refer to Table S1.

Fig. S3. Dendrogram of the cuticular hydrocarbon profiles of workers (Ward method, Euclidian distance). The first letter refers to colony origin (A, B or C), and the second refers to the group of workers (B=internal worker with actinobacteria, S = internal worker without actinobacteria, and E = external workers).

Table 1

ACCEPTED MANUSCRIPT

		EXT		INB		IN0		All ants		Bacteria	Gelose
N°	name	mean	SE	mean	SE	mean	SE	mean	SE		
1	C27	0.60	0.05	0.99	0.34	0.85	0.08	0.82	0.12	10.11	5.57
2	11-+13-MeC27	1.00	0.09	1.35	0.22	1.62	0.13	1.33	0.09	4.03	2.61
3	3-MeC27	0.67	0.07	0.66	0.12	0.96	0.14	0.77	0.07	6.24	3.69
4	C28	0.72	0.08	1.23	0.14	2.13	0.25	1.37	0.13	13.87	11.25
5	12-meC28	3.96	0.22	4.39	0.32	4.13	0.28	4.17	0.16	6.52	2.21
6	10,14-DiMeC28	0.86	0.12	1.00	0.18	0.70	0.08	0.85	0.08	3.73	0.47
7	C29	3.58	0.51	2.65	0.33	2.47	0.31	2.89	0.23	6.44	5.26
8	11-+13-+15-C29	32.33	1.63	30.89	1.17	30.90	0.72	31.36	0.69	12.44	24.23
9	11,15-+13,17-DiMeC29	12.03	0.59	12.85	0.78	12.82	0.69	12.57	0.40	5.22	11.03
10	5-MeC29	0.12	0.05	0.17	0.06	0.08	0.03	0.13	0.03	0.00	0.00
11	C30	0.99	0.06	0.90	0.05	0.92	0.07	0.94	0.04	3.70	2.29
12	14-+15-MeC30	6.46	0.19	6.50	0.13	6.59	0.09	6.52	0.08	4.96	5.86
13	12,16-DiMeC30	4.41	0.42	4.65	0.28	4.59	0.29	4.55	0.19	0.00	5.23
14	C31	1.16	0.10	1.05	0.06	1.10	0.09	1.10	0.05	0.00	1.21
15	Alcohol	0.91	0.12	0.91	0.08	0.88	0.13	0.90	0.06	0.00	0.00
16	11-+13-MeC31	10.84	0.46	10.69	0.92	10.46	0.60	10.66	0.40	8.50	9.27
17	11,15-+13,1-DiMe7C31	10.97	0.85	10.87	0.44	10.84	0.44	10.89	0.34	5.89	9.81
18	C32	0.50	0.06	0.53	0.03	0.38	0.06	0.47	0.03	0.00	0.00
19	10-+11-+12-MeC32	0.34	0.06	0.32	0.04	0.39	0.18	0.35	0.06	4.47	0.00
20	11-+13-MeC33	0.98	0.15	1.13	0.14	0.97	0.17	1.03	0.09	3.87	0.00
21	x,y-DiMeC33	1.81	0.27	1.81	0.16	1.44	0.16	1.68	0.11	0.00	0.00
22	11-+13-MeC35	2.37	0.09	2.39	0.18	2.61	0.13	2.46	0.08	0.00	0.00
23	11,15-DiMeC35	2.25	0.24	1.99	0.19	2.06	0.19	2.09	0.12	0.00	0.00
24	x,y-DiMeC35	0.12	0.03	0.09	0.03	0.11	0.04	0.11	0.02	0.00	0.00
	Total	100.00		100.00		100.00		100.00		100.00	100.00
		n=	17		18		18		53	1	1

Fig. 1

ACCEPTED MANUSCRIPT

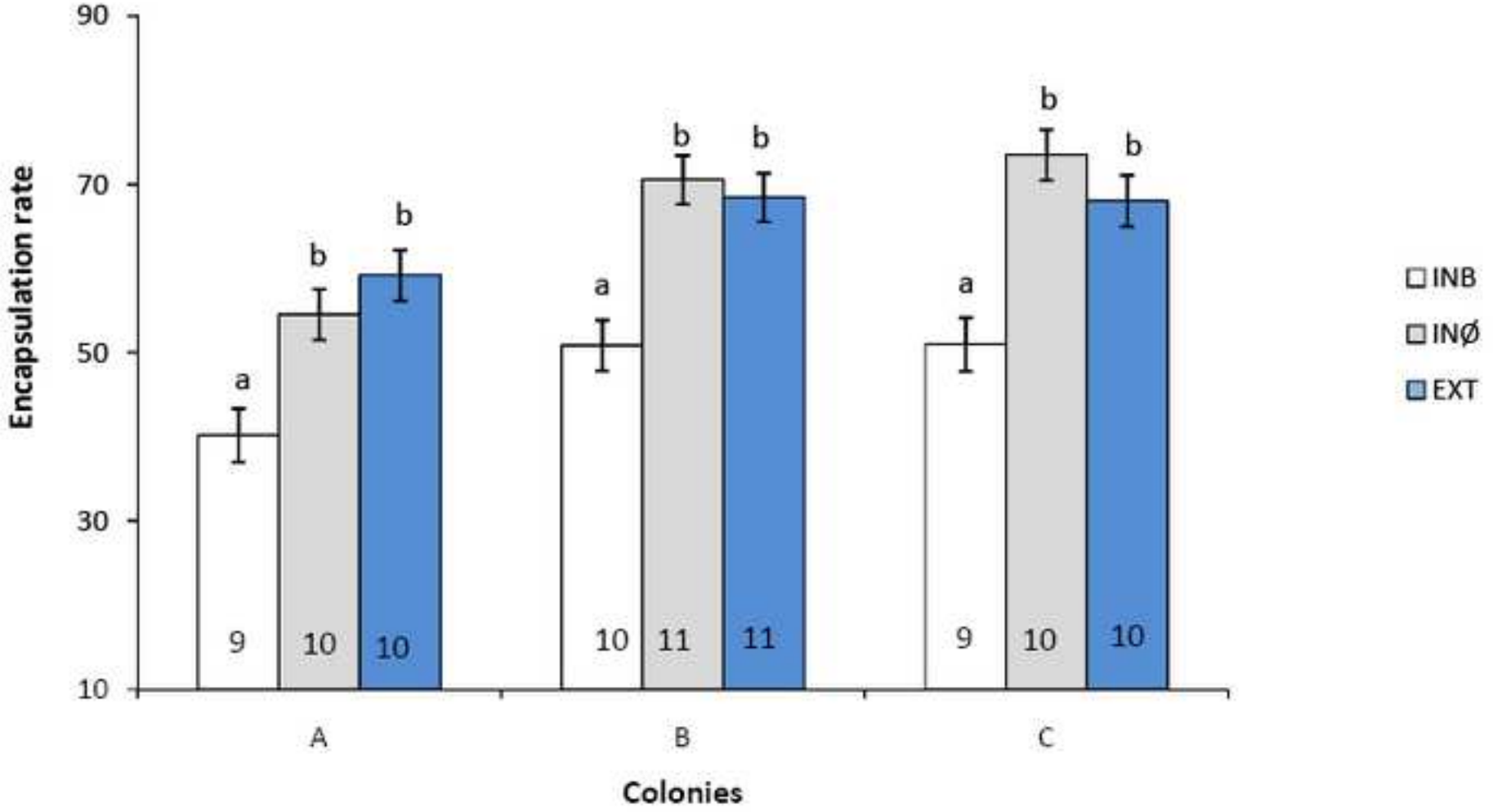


Fig. 2

ACCEPTED MANUSCRIPT

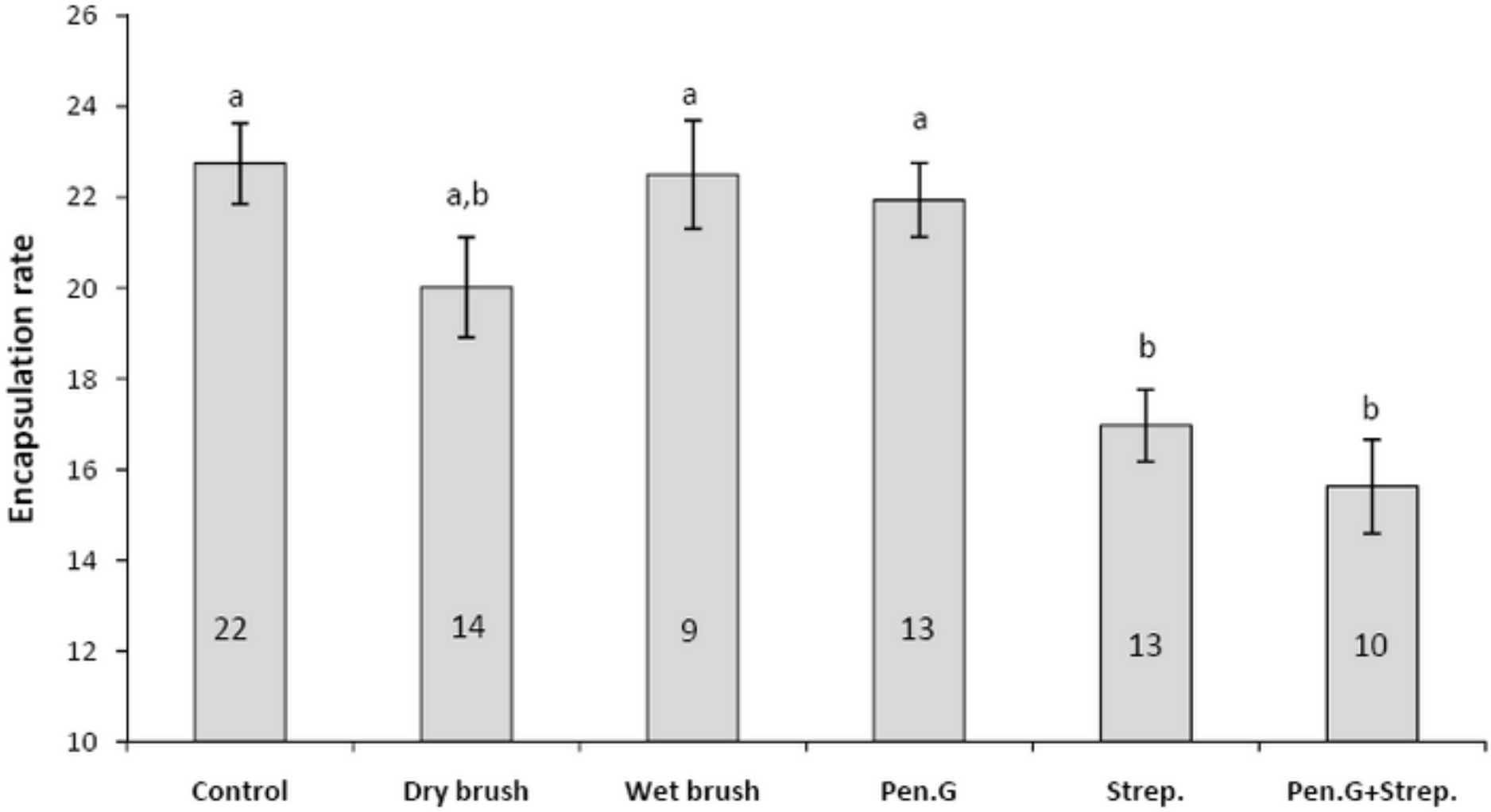


Fig. 3

ACCEPTED MANUSCRIPT

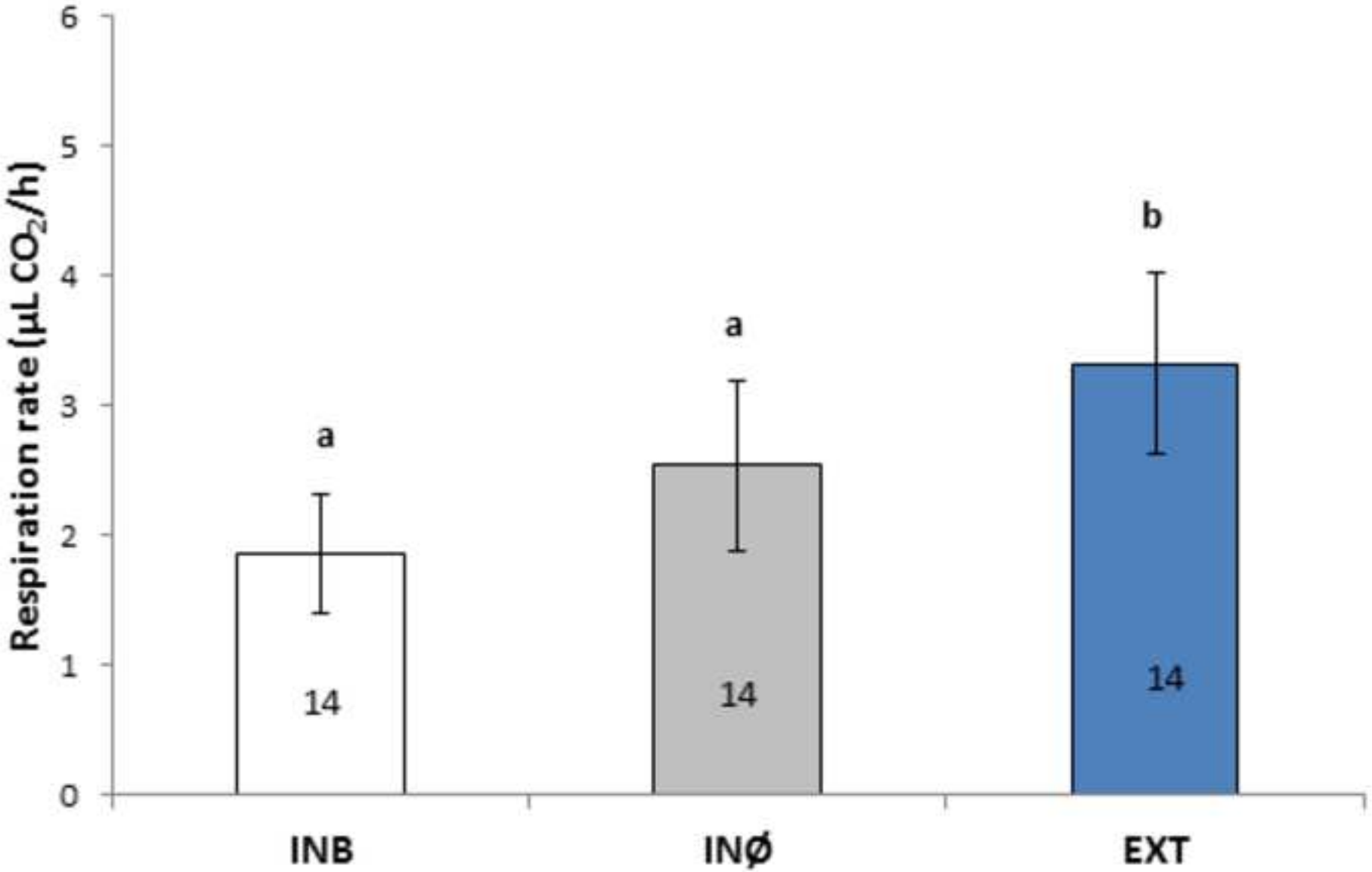


Fig. S1

ACCEPTED MANUSCRIPT

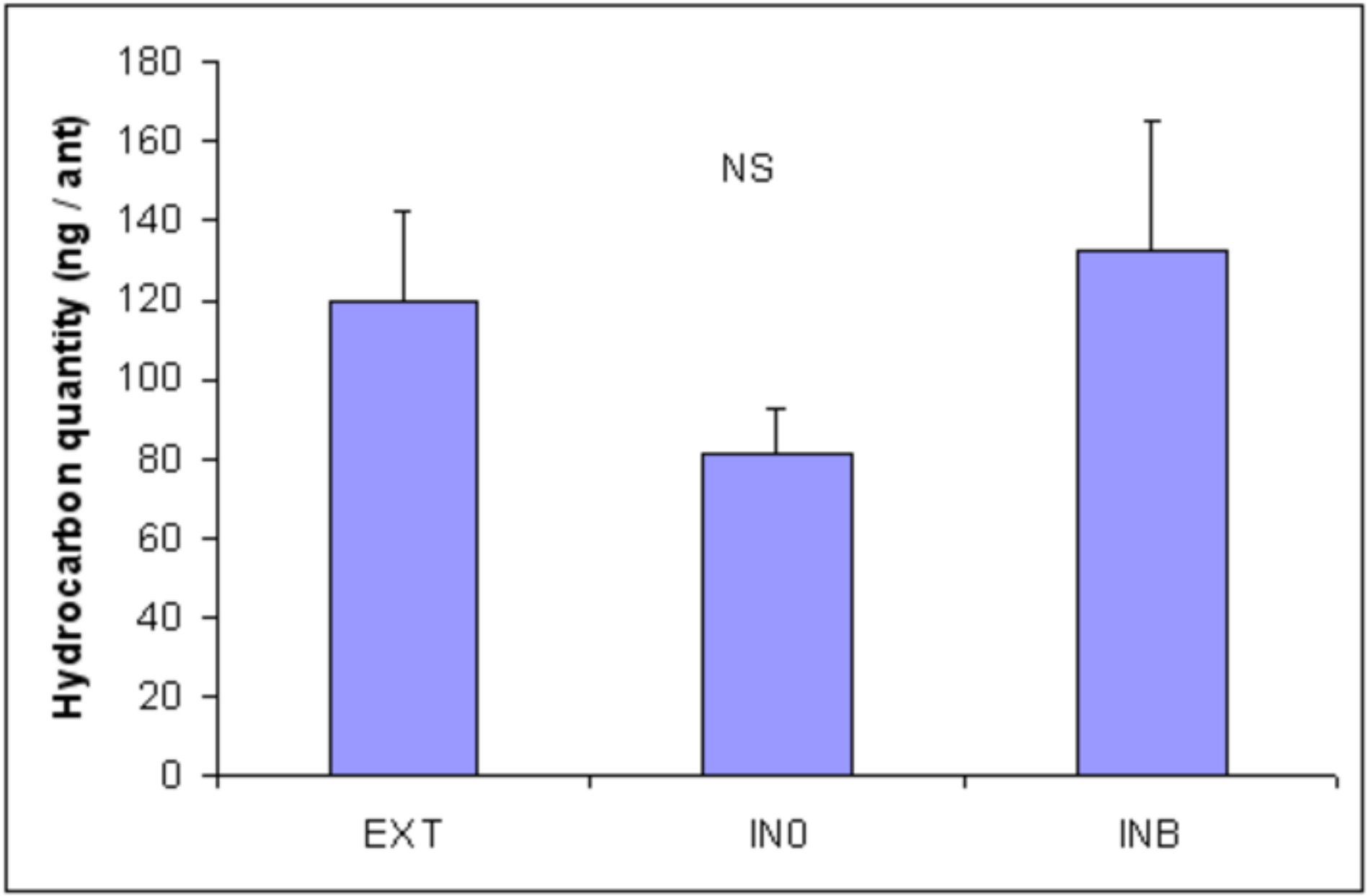
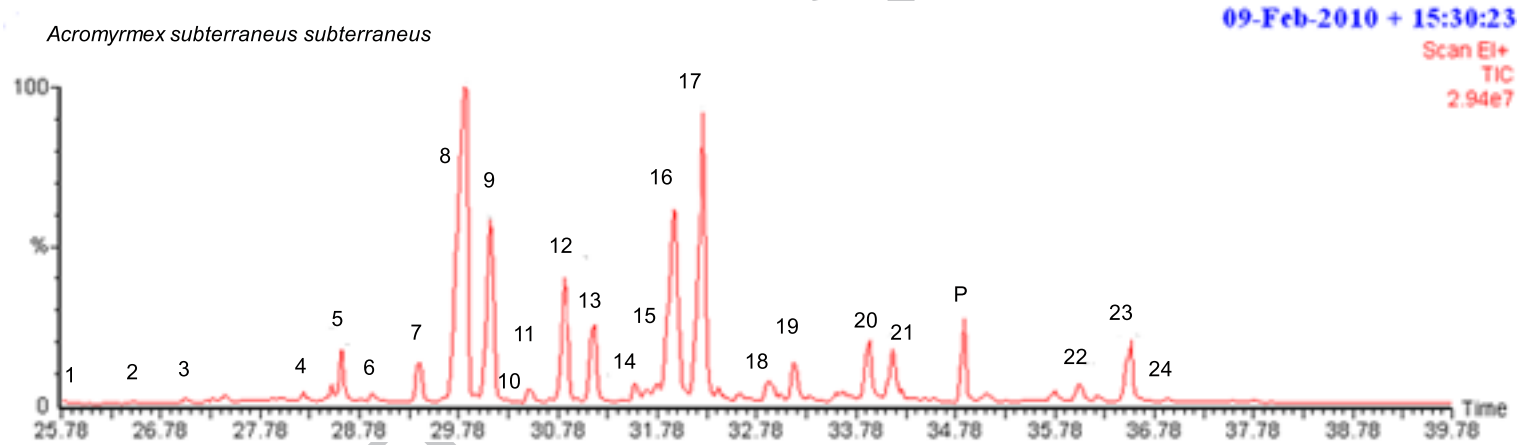


Fig. S2

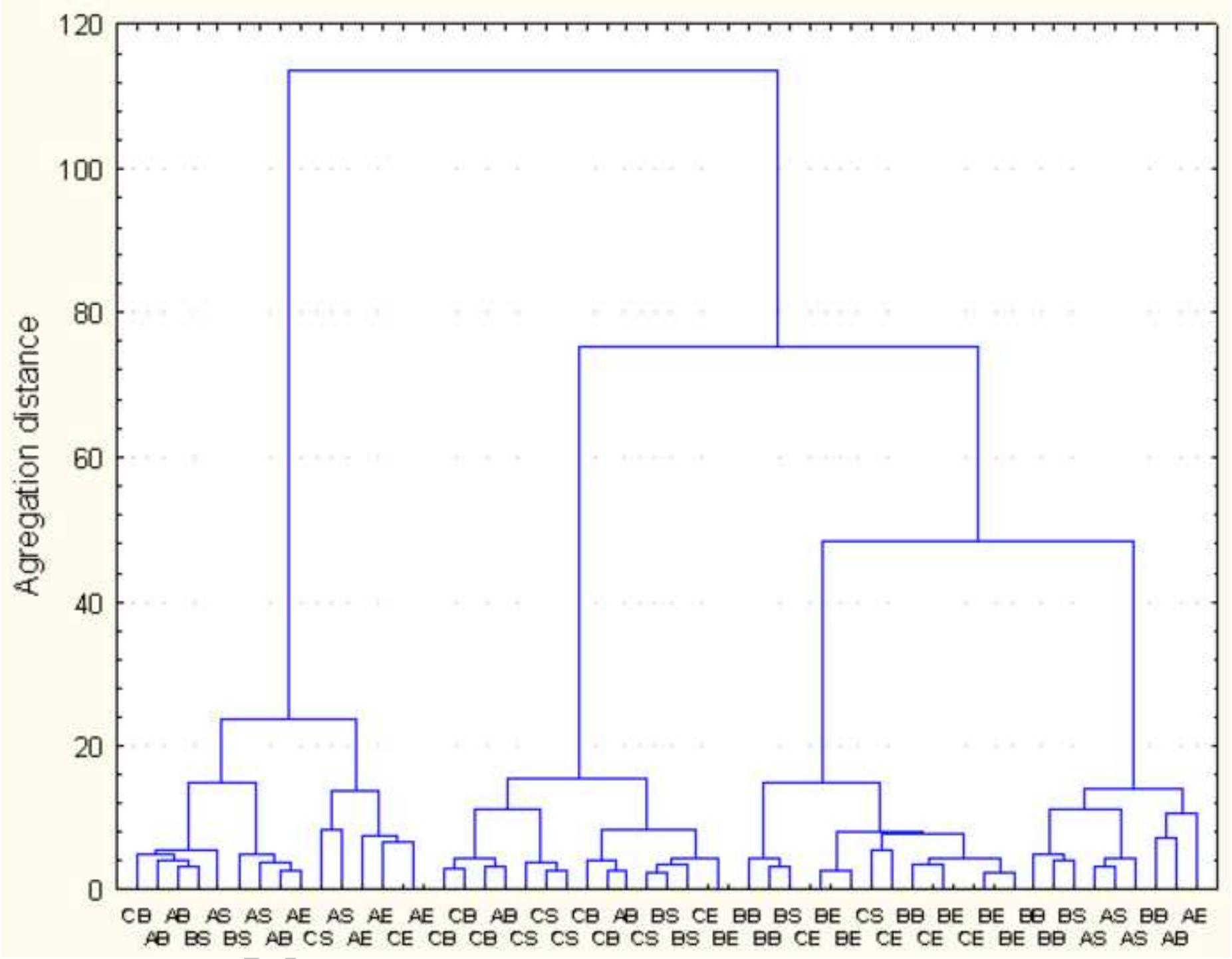
ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT

Fig. S3

ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT

Highlight :

<*Acromyrmex subterraneus subterraneus* workers covered with actinobacteria have lower encapsulation response<

ACCEPTED MANUSCRIPT